INFECTION AND IMMUNITY, Oct. 1992, p. 4322–4327
0019-9567/92/104322-06$02.00/0

Vol. 60, No. 10

Hemagglutination and Adherence to Plastic by *Staphylococcus epidermidis*

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Received 27 May 1992/Accepted 27 July 1992

***Staphylococcus epidermidis* is an important nosocomial pathogen responsible for intravenous catheter-related bacteremia and infections of other prosthetic medical devices. We found that the ability of *S. epidermidis* to hemagglutinate erythrocytes correlated with the adherence of bacteria to plastic and to intravenous catheters. *S. epidermidis* isolates responsible for prosthetic-valve endocarditis ($n = 61$) and isolates from intravenous catheters ($n = 59$) were significantly more likely to cause hemagglutination than isolates from the skin of preoperative cardiac surgery patients ($n = 19$) ($P = 0.027$). *S. epidermidis* isolates ($n = 23$) recovered from the skin of patients 7 to 10 days after cardiac surgery were significantly more likely to exhibit hemagglutination than the preoperative isolates ($P = 0.015$). By a quantitative adherence assay, we also observed that the hemagglutination titer and number of species of erythrocytes agglutinated correlated directly with adherence to polystyrene ($P < 0.001$). In addition, hemagglutinating isolates were significantly more likely to be recovered in high number from intravenous catheters when semiquantitative catheter culture techniques were used ($P < 0.001$). We speculate that hemagglutinin(s) either plays a direct role in adherence to polymers and thus prosthetic-device infection or serves as an easily demonstrable marker for adherence-prone isolates.**

Staphylococcus epidermidis, a generally avirulent commensal organism of human skin, is the principal etiologic agent of infections of peripheral and central venous catheters, prosthetic heart valves, artificial joints, and other prosthetic devices (29). Despite its importance as a nosocomial pathogen, relatively little is known about the pathogenesis of these infections or the virulence determinants of this organism.

Hemagglutination is the aggregation of erythrocytes caused by bacteria adhering to two or more erythrocytes. Hemagglutination is a commonly used assay to demonstrate bacterial adherence and, in gram-negative organisms, has been shown to reflect specific lectin-receptor interactions that are important in the pathogenesis of disease (2, 23, 27, 31). Previous reports of hemagglutination by staphylococci have concentrated primarily on the uropathogen *Staphylococcus saprophyticus* (3, 16, 18). Studies of hemagglutination by *S. epidermidis* have not utilized clinically relevant isolates and have tested few types of erythrocytes (3, 18). Adherence to biomaterials by *S. epidermidis* has not been previously associated with hemagglutination. We have observed that many clinically significant isolates of *S. epidermidis* agglutinate the erythrocytes of humans, sheep, and other animal species. In addition, we have demonstrated that there is a strong correlation between hemagglutination and adherence to plastic and intravenous catheters. We hypothesize that the hemagglutinin may play a role in the pathogenesis of prosthetic-device infections or may serve as a surrogate marker for adherence-prone isolates.

(Part of this research was presented at the 31st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., 1991 [29a].)

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MATERIALS AND METHODS

Bacteria. Several collections of coagulase-negative staphylococci were studied. One group consisted of isolates, previously characterized (20), from patients with prosthetic-valve endocarditis (PVE) from a wide geographic area in the United States. The second group contained isolates recovered from intravenous catheters at the Medical College of Virginia in Richmond, Va. The third group of isolates was obtained preoperatively and postoperatively from the skin of cardiac surgery patients at the Medical College of Virginia. These preoperative isolates have previously been shown to be methicillin susceptible, while the postoperative isolates are predominantly methicillin resistant. Furthermore, the preoperative and postoperative isolates all differed from one another by plasmid pattern analysis (37). Reference strains of *S. epidermidis*, RP62A (ATCC 35984) and RP62NA, which have been previously characterized with regard to their adherence properties (5), were supplied as a gift (G. Pier, Boston, Mass.). In addition, G. Pier supplied 11 strains of *S. epidermidis* that have been characterized as positive for the presence of polysaccharide-adhesin (PS/A) (34).

Coagulase-negative staphylococci were identified to species level as described by Kloos and Schleifer (22). Briefly, coagulase-negative staphylococci were identified as *S. epidermidis* if they produced acid aerobically from sucrose and maltose and did not ferment xylose or trehalose.

Media. Bacteria were maintained on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.). Trypticase soy broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.) was used in the hemagglutination and adherence assays.

Semiquantitative cultures. Semiquantitative catheter cultures were performed by the method described by Maki et al. (24). For the purposes of statistical analysis, counts for bacterial colonies that were noted to be too numerous to count were recorded as 101 CFU.

Erythrocytes. Suspensions (1% each) of human A, human O, sheep, horse, ox, pig, rabbit, guinea pig, and chicken

erythrocytes (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) were used in the hemagglutination assay. Bovine serum albumin (Sigma Chemical Co.) was added to a concentration of 0.1% in order to prevent nonspecific agglutination of the erythrocytes.

Hemagglutination. Hemagglutination was assessed in a manner similar to the method described by Hovelius and Mardh (18). Bacteria from an overnight incubation at 37°C in Trypticase soy broth were harvested by centrifugation and washed once with PBS. Bacterial concentrations were determined by McFarland turbidity standards (14) and bacterial plate counts. The bacterial suspensions were adjusted to a McFarland standard of 3.0, which correlated with approximately 10^8 bacteria per ml. The bacterial suspensions were diluted 1:10 in PBS. The hemagglutination assay was performed with 96-well (U-shaped) microtiter plates (Costar, Cambridge, Mass.). Twofold serial dilutions of the 10% bacterial suspension were performed in the microtiter plates to give a total volume of 50 μl per well. Next, 50 μl of the 1% erythrocyte suspension was added to each well. The plates were sealed with cellophane, shaken to ensure even mixing of the bacteria and erythrocytes, and then incubated at room temperature for 2 h. Hemagglutination was rated on a scale from 0 to +++ (0 hemagglutination was recorded when a compact pellet of erythrocytes was observed, + was defined as a loose pellet with a surrounding halo of hemagglutination, ++ was defined as diffuse hemagglutination with some poorly organized sedimentation of erythrocytes, and +++ was recorded when diffuse hemagglutination was observed). All tests were performed in triplicate.

Adherence. Adherence to polystyrene was assessed by the qualitative and quantitative methods described by Christensen et al. (6, 7).

Statistical analysis. Comparisons between the various strain collections of the ability of staphylococci to hemagglutinate were made by the chi-square test (10). The degree of association between hemagglutination and adherence to plastic was assessed by linear regression analysis (10). The hemagglutination titer was linearized by assigning the numbers 0 to 8 to titers 0 to 1:1,280, respectively. Significance was determined by use of Student's *t* test (two-tailed) (10).

RESULTS

Hemagglutination: PVE isolates. Figure 1 shows the hemagglutination assay for five isolates of hemagglutination-positive *S. epidermidis* and five strains of hemagglutination-negative *S. epidermidis*. Table 1 summarizes the hemagglutination data for the PVE isolates. Seventy-one isolates of coagulase-negative staphylococci were examined. Sixty-one of the isolates were identified as *S. epidermidis*. Hemagglutination was observed to be a common phenotype among these *S. epidermidis* isolates, with 63.9% causing hemagglutination of one or more species of erythrocyte. For specific animal species of erythrocytes, the prevalence of hemagglutination ranged from a high of 54.1% for ox erythrocytes to a low of 32.8% for horse erythrocytes. A total of 18 (29.5%) of the isolates of *S. epidermidis* agglutinated all 9 types of erythrocytes, while 22 (36.1%) of the isolates did not agglutinate any of the tested types of erythrocytes. Only 40% of the non-*S. epidermidis* coagulase-negative staphylococci caused hemagglutination of any of the types of erythrocytes. No more than three types of erythrocytes were agglutinated by any one of these isolates, and in all cases, the degree of hemagglutination was relatively weak (bacterial dilutions of no greater than 1:20). Non-*S. epidermidis* species causing PVE included *S. warneri*, *S. haemolyticus*, *S. hominis*, and *S. intermedius*.

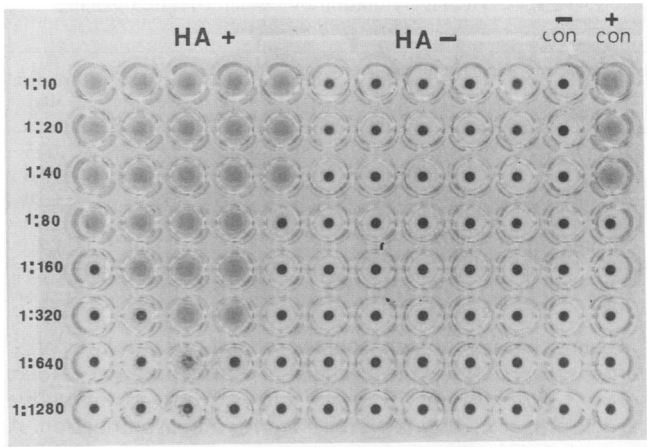

FIG. 1. Typical appearance of hemagglutination assay. The five columns of wells on the left side of the plate are hemagglutination-positive (HA +) isolates; the next five columns of wells are hemagglutination-negative (HA −) isolates. Bacterial dilutions are shown at left, ranging from 1:10 to 1:1,280. Negative control (con −), *S. aureus* RN4220; positive control (con +), *S. epidermidis* SE5.

Hemagglutination: venous-catheter isolates. Seventy-four isolates of coagulase-negative staphylococci recovered from intravenous catheters were studied. Fifty-nine of the isolates were identified as *S. epidermidis*. Table 2 summarizes the hemagglutination data for the intravenous-catheter isolates. Eighty-three percent of the *S. epidermidis* strains hemagglutinated the erythrocytes of one or more of the five animal species of erythrocytes tested (five different types of blood instead of nine were tested because of difficulties in obtaining lyophilized erythrocytes from the supplier). Thirty-three (55.9%) of the isolates agglutinated all five types of erythrocytes, while 11 (18.6%) of the isolates did not agglutinate any of the tested types of erythrocytes. For specific types of erythrocytes, the rate of agglutination ranged from a high of 78% for guinea pig erythrocytes to a low of 59% for human A erythrocytes. Of the 15 non-*S. epidermidis* isolates of coagulase-negative staphylococci, only 33% of the isolates agglutinated the erythrocytes of any of the five types of animal blood cells tested. These isolates each agglutinated no more than a single type of erythrocyte, and agglutination was observed only at low bacterial titers (dilutions of no greater than 1:40).

TABLE 1. Hemagglutination by PVE isolates of *S. epidermidis*

Blood type or source of erythrocyte	Hemagglutination prevalence (%) (n = 61 strains)	High titer	Low titer	Geometric mean titer	Median titer
Human A	26 (43)	1:1,280	0	1:60	1:20
Human O	31 (51)	1:1,280	0	1:85	1:40
Sheep	26 (43)	1:1,280	0	1:56	1:80
Horse	20 (33)	1:160	0	1:38	1:40
Ox	33 (54)	1:640	0	1:35	1:20
Pig	25 (41)	1:640	0	1:43	1:40
Rabbit	24 (39)	1:1,280	0	1:90	1:80
Guinea pig	34 (56)	1:1,280	0	1:78	1:40
Chicken	21 (34)	1:320	0	1:32	1:40

TABLE 2. Hemagglutination by catheter-related isolates of *S. epidermidis*

Blood type or source of erythrocyte	Hemagglutination prevalence (%) (n = 59 strains)	High titer	Low titer	Geometric mean titer	Median titer
Human A	35 (39)	1:320	0	1:47	1:15
Sheep	45 (76)	1:1,280	0	1:102	1:80
Pig	38 (64)	1:160	0	1:54	1:20
Guinea pig	46 (78)	1:1,280	0	1:93	1:70
Chicken	37 (63)	1:80	0	1:46	1:10

Hemagglutination: skin isolates. A total of 19 isolates of *S. epidermidis* recovered from the skin of preoperative cardiac surgery patients and 23 skin isolates from postoperative cardiac surgery patients were tested for hemagglutination. A total of 6 (31.6%) of the preoperative isolates exhibited hemagglutination compared with 17 (73.9%) of the postoperative isolates (P = 0.015 [chi-square test]).

Hemagglutination: RP62A, RP62NA, and PS/A strains. Because sheep erythrocytes yielded the least ambiguous results (hemagglutination scores of 0 or +++, with few results of + or ++), we used them to test the hemagglutination capability of strains of *S. epidermidis* previously characterized for adherence and slime production. RP62A agglutinated sheep erythrocytes at a low titer (1:10), whereas RP62NA did not cause hemagglutination. Eight strains of *S. epidermidis* that were characterized as PS/A (+) and slime (−) did not cause hemagglutination of sheep erythrocytes even when the bacterial suspension was concentrated 10-fold. Three strains of *S. epidermidis* that were defined as PS/A (+) and slime (+) also failed to hemagglutinate sheep erythrocytes.

Adherence: PVE isolates. Figure 2 shows an example of the correlation between hemagglutination of sheep erythrocytes and adherence to polystyrene by the two adherence assays.

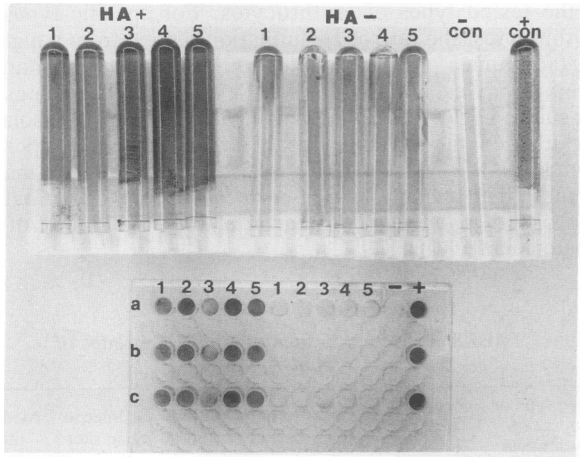

FIG. 2. Correlation between hemagglutination of sheep erythrocytes and qualitative and quantitative adherence assays. Isolates of *S. epidermidis* are the same isolates as those shown in Fig. 1. Quantitative assay is shown in triplicate in rows a, b, and c. Average ODs (λ = 570 nm) of quantitative wells for hemagglutination-positive (HA +) strains 1 to 5 are 1.622, 2.0, 1.072, 2.0, and 2.0; those for hemagglutination-negative (HA −) strains 1 to 5 are 0.079, 0.062, 0.069, 0.087, 0.068; and those for positive control (+ con) and negative control (− con) are 2.0 and 0.061, respectively. Negative control, *S. aureus* RN4220; positive control, *S. epidermidis* SE5.

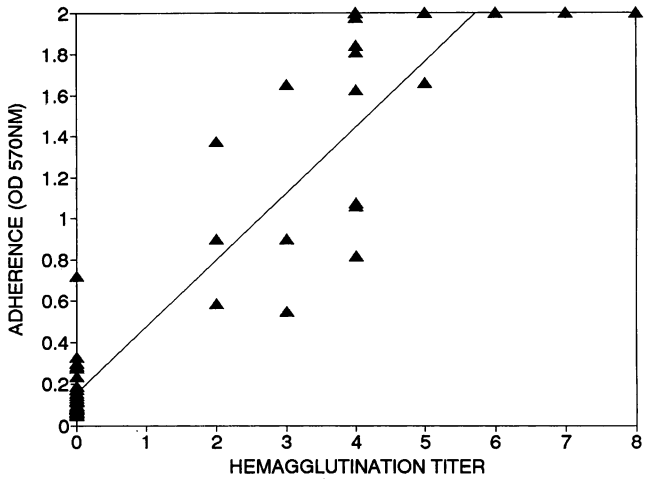

FIG. 3. Linear-regression plot of hemagglutination titer versus adherence for PVE strains of *S. epidermidis*. Regression equation: (adherence) = (0.178) (hemagglutination) + (0.328); $P < 0.001$ and r = 0.923. Hemagglutination titers 1 to 8 represent serial bacterial dilutions of 1:10 to 1:1,280, respectively.

The 10 strains of hemagglutination-positive and hemagglutination-negative *S. epidermidis* are the same 10 strains shown in Fig. 1. Because other investigators have shown the qualitative adherence assay to be poorly reproducible (7), the quantitative adherence assay was used for the analysis of adherence. A strong correlation between hemagglutination and adherence to plastic was observed. Figure 3 shows a plot of hemagglutination versus adherence for the PVE isolates of *S. epidermidis*. A linear regression analysis was performed, and the equation of the regression line for these data is as follows: (adherence optical density [OD]) = (0.1796) (hemagglutination) + 0.328. The correlation coefficient is 0.923. Using Student's t test to determine significance for the association between hemagglutination and adherence yields $P < 0.001$. If the number of different types of blood cells agglutinated rather than the titer of agglutination is considered, the association between agglutination and adherence remains statistically significant. The equation for the regression line is as follows: (adherence OD) = (0.11) (number of species of erythrocytes agglutinated) + 0.169. The correlation coefficient is 0.879, and $P < 0.001$.

Adherence: intravenous-catheter isolates. The relationship between hemagglutination of sheep erythrocytes and adherence to polystyrene for the intravenous-catheter isolates of *S. epidermidis* is shown in Fig. 4. The linear regression equation is as follows: (adherence OD) = (0.277) (hemagglutination) + 0.22. The correlation coefficient is 0.87, and $P < 0.001$. A significant association between hemagglutination of sheep erythrocytes and the semiquantitative catheter cultures is also observed. The linear regression equation for these data is as follows: (semiquantitative catheter culture) = (47.6) (hemagglutination) + 5.5. The correlation coefficient is 0.43, and $P < 0.001$. The linear-regression plot for the semiquantitative catheter cultures versus adherence was also examined. The equation of the regression line is (adherence OD) = (0.498) (semiquantitative catheter culture) + 0.492. The correlation coefficient is 0.49, and $P < 0.001$.

Adherence: skin isolates. The relationship between hemagglutination and adherence to polystyrene for the perioperative skin isolates was similar to that observed for the PVE isolates and catheter-related isolates. Strains causing hem-

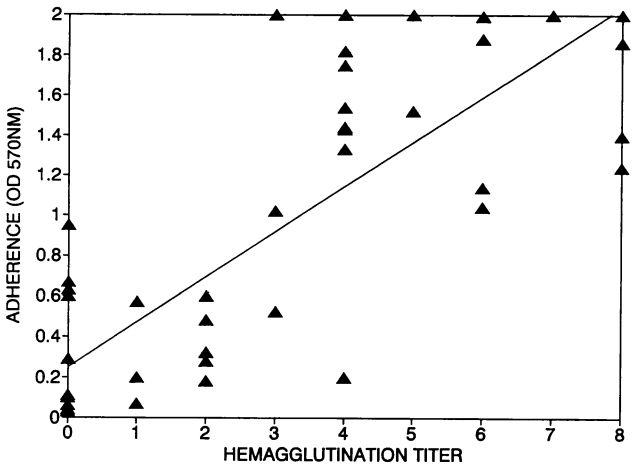

FIG. 4. Linear-regression plot of hemagglutination titer versus adherence for catheter-associated strains of *S. epidermidis*. Regression equation: (adherence) = (0.277) (hemagglutination titer) + (0.22); $P < 0.001$ and $r = 0.87$. Hemagglutination titers 1 to 8 represent serial bacterial dilutions of 1:10 to 1:1,280, respectively.

agglutination were significantly more adherent ($P < 0.001$) for both preoperative and postoperative isolates. The linear regression equation for the preoperative isolates is as follows: (adherence OD) = (0.22) (hemagglutination) + (0.22); $r = 0.923$. The linear regression equation for the postoperative isolates is (adherence OD) = (0.19) (hemagglutination) + (0.458); $r = 0.796$.

DISCUSSION

Hemagglutination by gram-negative organisms has been intensively studied as a model of adherence. The hemagglutinins are often proteins located on filamentous organelles that interact specifically with carbohydrate receptors on the surfaces of erythrocytes and other eukaryotic cells. These lectin-receptor interactions are important for the adherence of bacteria to mucosal surfaces, which is recognized as a vital first step in the pathogenesis of many diseases (2). In contrast, hemagglutination by staphylococci has not generated as much interest; studies have primarily concerned themselves with *S. saprophyticus*. The hemagglutinin of *S. saprophyticus* appears to be a protein that interacts specifically with oligosaccharides on the surfaces of sheep erythrocytes (13). In this regard, it is of interest to note evidence of fimbrialike surface structures on *S. saprophyticus* (30) and *S. epidermidis* (33). Further studies to elucidate the nature of the *S. epidermidis* hemagglutinin are required.

The question of whether an association exists between hemagglutination by *S. epidermidis* and virulence is intriguing. *S. epidermidis* isolates from patients with PVE were significantly more likely to cause hemagglutination than isolates from preoperative cardiac surgery patients ($P = 0.027$ [chi-square test]). A similar association was seen when *S. epidermidis* isolates recovered from intravenous catheters were compared with the skin isolates ($P = 0.00014$ [chi-square test]). What makes the hypothesis of a link between hemagglutination and virulence more tenable is the observation that *S. epidermidis* isolates which hemagglutinate at greater bacterial dilutions or which hemagglutinate greater numbers of animal species of erythrocytes are significantly more adherent to plastic. However, it is unknown whether the hemagglutinin plays a direct role in the adherence process or serves as a marker for adherence-prone isolates. It is also unknown whether the administration of antibiotics plays a role in selecting for hemagglutination-positive, and thus more adherent, strains of *S. epidermidis*. The postoperative isolates were obtained 7 to 10 days after cardiac surgery. All of these patients received prophylactic antibiotics (primarily cefazolin). Most of the preoperative isolates were susceptible to methicillin (89.5%), whereas the majority of the postoperative isolates were resistant to methicillin or gentamicin (95%). Thus, there may be a link between antibiotic resistance and hemagglutination. The association between antibiotic resistance and a putative virulence determinant of *S. epidermidis* is not novel. Christensen et al. described a link between the colony morphology of *S. epidermidis* when grown on Memphis agar, slime production, and resistance to β-lactam antibiotics (4).

It is accepted that bacterial adherence to biomaterials is a crucial first step in the pathogenesis of prosthetic-device infection. A number of factors influence an organism's ability to adhere to biomaterials. These factors include characteristics of the bacterium, the surface structure of the biomaterial, and the nature of the ambient milieu. Initial attachment of bacteria to the surface of a biomaterial is a reversible process that is influenced by surface charge, van der Waals forces, and hydrophobic interactions (12). It would appear from the large number of prosthetic-device infections that are caused by *S. epidermidis* that this organism has some unique attribute(s) that enables it to cause infection more readily than other competing organisms. This factor may be the ability to adhere to biomaterials more avidly than other bacteria.

Investigation concerning the adherence of *S. epidermidis* to biomaterials has concerned itself primarily with the role of the extracellular polysaccharide or slime. Despite intensive study, the role of slime in the pathogenesis of disease and even its composition remain debated (8). Currently, extracellular slime is thought to play a role in the later stages of adherence and persistence of infection. It may serve as an ion-exchange resin to optimize the local nutritional environment, prevent penetration of antibiotics into the macrocolony, and protect the bacterium from phagocytic host defense cells (11). Peters et al. have shown by electron microscopy studies that extracellular polysaccharide appears in the later stages of attachment and is not present during the initial phase of adherence (28). Hogt et al. demonstrated that removal of the extracellular slime layer by repeated washing did not diminish the ability of *S. epidermidis* to adhere to biomaterials (17). Thus, it is unclear what role extracellular polysaccharide plays in the initial stages of adherence.

S. epidermidis is most frequently isolated in prosthetic-device infections when the biomaterial surface is a polymer or contains a polymer, such as those of intravascular catheters, vascular grafts, or prosthetic joints (11). Hsieh and Merry showed that *S. epidermidis* adheres to polymers at a higher rate than *Staphylococcus aureus* does (19). We have examined several strains of *S. aureus* and have not observed isolates causing hemagglutination at high titers (28a). This may in part explain the differences in adherence between these two closely related species of staphylococci that are observed. In addition, numerous studies have shown that human plasma proteins enhance the adherence of *S. aureus* to medical polymers (15, 35, 36). This relationship is less clear with coagulase-negative staphylococci (26). After implantation, biomedical devices are rapidly coated by a conditioning film consisting of host glycoproteins (1). Fimbriae

and other proteinaceous adhesins may play a crucial role in the adherence process by reacting specifically with host receptors found in this conditioning film, or they may exert their influence by nonspecific hydrophobic and electrostatic interactions (32). It is via this mechanism that we hypothesize the hemagglutinin of *S. epidermidis* to exert its influence in the adherence of this organism to polymer-containing biomaterials. Because we have observed isolates of *S. epidermidis* which do not cause hemagglutination and yet which have resulted in well-documented cases of prosthetic-device infection and because we have observed that there are isolates that adhere to plastic but do not hemagglutinate, it seems likely that there is more than one factor that influences adherence. Perhaps the adherence properties described by other investigators, such as the PS/A described by Tojo et al. (34) or the proteinaceous adhesin described by Timmerman and colleagues (33), may explain these discrepancies. Further investigation to try to correlate these various adherence mechanisms should be made.

A particularly vexing problem frequently confronting clinicians is the difficulty in differentiating true cases of *S. epidermidis* bacteremia from contaminants of procurement. Numerous investigators have proposed guidelines for answering this crucial issue (9, 21, 25). Unfortunately, none is uniformly reliable. Although the hemagglutination test does not appear to offer a perfect solution, since we have observed clinically significant strains that do not hemagglutinate and there is a certain amount of variability in the hemagglutination titers in relation to adherence, it may complement the existing guidelines. Further investigation with well-defined collections of isolates complete with clinical correlation should be made.

In conclusion, we have demonstrated that hemagglutination is a common phenotypic trait of *S. epidermidis*. This trait appears to be more frequently observed among isolates responsible for infection. We have also shown that there is a significant correlation between hemagglutination and adherence to plastic. It is unknown whether this is a direct effect or simply an epiphenomenon, but it is not unreasonable to hypothesize that the hemagglutinin of *S. epidermidis* may play a major role in the adherence of this organism to polymer-containing biomaterials. Thus, the hemagglutinin of *S. epidermidis* may be a virulence factor in the pathogenesis of prosthetic-device infection or it may serve as an easily demonstrated marker for adherence-prone and therefore clinically relevant isolates.

ACKNOWLEDGMENTS

We thank R. Polk for assistance with the statistical analysis.

This work was supported by Public Health Service grants AI 21772 and F32 AI 08416 from the National Institutes of Allergy and Infectious Diseases and by an award from the Southern Medical Association.

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